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(12)(19)(CA) **Demande-Application**

(21)(A1) **2,198,645**
(22) 1997/02/27
(43) 1998/08/27

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(51) Int.Cl.[6] A61K 31/36, A01N 43/30

(54) **UTILISATION DU DILLAPIOL ET DE SES ANALOGUES ET DERIVES POUR AGIR SUR DES CELLULES MULTIRESISTANTES AUX MEDICAMENTS**

(54) **USE OF DILLAPIOL AND ITS ANALOGUES AND DERIVATIVES TO AFFECT MULTIDRUG RESISTANT CELLS**

(57) La méthode couverte par cette invention a trait à l'usage du dillapiol ou de ses analogues et dérivés pour agir sur les cellules exprimant une résistance à de nombreux médicaments (multirésistance). Dans une matérialisation, le dillapiol ou ses analogues et dérivés sont utilisés seuls comme agents exerçant un effet toxique direct sur les cellules qui présentent un phénotype de multirésistance. Dans une autre, le dillapiol ou ses analogues et dérivés sont utilisés comme chimiosensibilisants à certains agents chimiques, comme les agents chimiothérapeutiques, les insecticides et la nicotine, afin d'accroître l'efficacité de ces agents. Cette méthode est utile pour le traitement des pathologies ainsi que des processus physiologiques dans lesquels intervient la multirésistance aux médicaments, comme certains cancers ou maladies (p. ex. le paludisme), différents états de tolérance ou de résistance (p. ex. aux insecticides), et la dépendance (vis-à-vis de la nicotine et des opiacés).

(57) The method of this invention entails the use of dillapiol or its analogues and derivatives to affect cells expressing multidrug resistance (MDR) activity. In one aspect, dillapiol or its analogues and derivatives are used alone as agents that exert a direct toxic effect on cells presenting an MDR phenotype. In another aspect, dillapiol or its analogues and derivatives are used as chemosensitizers to chemical agents, such as chemotherapeutic agents, insecticides, and nicotine, in order to increase the efficacy of these agents. This method is useful for treating pathologies and physiologies in which MDR plays a role, such as certain cancers and diseases (eg. malaria), different states of tolerance or resistance (eg to insecticides), and addiction (eg. to nicotine and opiates).

ABSTRACT

The method of this invention entails the use of dillapiol or its analogues and derivatives to affect cells expressing multidrug resistance (MDR) activity. In one aspect, dillapiol or its analogues and derivatives are used alone as agents that exert a direct toxic effect on cells presenting an MDR phenotype. In another aspect, dillapiol or its analogues and derivatives are used as chemosensitizers to chemical agents, such as chemotherapeutic agents, insecticides, and nicotine, in order to increase the efficacy of these agents. This method is useful for treating pathologies and physiologies in which MDR plays a role, such as certain cancers and diseases (eg. malaria), different states of tolerance or resistance (eg to insecticides), and addiction (eg. to nicotine and opiates).

THE USE OF DILLAPIOL AND ITS ANALOGUES AND DERIVATIVES TO AFFECT MULTIDRUG RESISTANT CELLS

1. FIELD OF THE INVENTION

The present invention relates to a method of affecting cells expressing multidrug resistance (MDR) activity.

2. BACKGROUND OF THE INVENTION

2.1 Multidrug Resistance

Multidrug Resistance (MDR) is the process whereby cells become resistant to structurally diverse chemotherapeutic agents following exposure to a single drug (Riordan and Ling, (1985) *Pharmol. Ther.* 28:51-75). The MDR phenotype is characterized by decreased intracellular accumulation of drugs or other chemical substances and by cross resistance to other structurally- and functionally-unrelated drugs or substances. The hallmark and clinically devastating aspect of MDR-based resistance is that the resistance extends to a wide range of drugs.

MDR is observed in a wide range of settings, ranging from the clinical setting to pest control management. In general, MDR can be observed whenever chemical substances are used to affect cells or organisms, and cells develop a resistance to the chemical agent. Examples include such diverse chemical substances as chemotherapeutic agents, insecticides, and nicotine. MDR occurs in pathologies such as certain cancers and diseases (eg. malaria), in certain states of tolerance or resistance (eg. to insecticides), and in addictions (eg. to nicotine and opiates).

A number of mechanisms have been shown to be involved in the MDR phenotype: a decreased

uptake of drugs; an increased energy-dependent efflux of drugs from the cell; a lower affinity to intracellular binding sites; and a slower conversion of drugs to alkali-labile materials as compared to drug-sensitive cells (Gottesman *et al.*, *In* Caplan (ed.) *Cell Biology and Membrane Transport Processes* (New York: Academic Press, 1994) 41:3-15).

One mechanism by which cells can develop resistance to an array of structurally-diverse drugs involves an ATP-binding cassette (ABC) superfamily of transport proteins (Higgins (1992) *Ann. Rev. Cell Biol.* 8:67; Hyde *et al.*, (1990) *Nature* 346:362). Members of the ABC superfamily bind ATP, using the free energy of ATP hydrolysis to drive particular biological reactions. These transport proteins are components of an active transport system that mediates the transport of molecules across the cytoplasmic membrane of cells. Members of this ABC superfamily, which share considerable sequence homology, include the following: the eukaryotic MDR P-glycoprotein; the protein pfMDR implicated in chloroquine resistance of the malarial parasite; the product of the cystic fibrosis gene CFTR, a transmembrane conductance regulator that controls chloride ion fluxes; the product of the White locus of Drosophila; prokaryotic proteins associated with membrane transport, cell division, nodulation, and DNA repair; and the STE-6 gene product that mediates export of yeast α-factor mating pheromone.

The MDR P-glycoprotein (P-gp) is a high molecular weight (150-170 kilodalton) eukaryotic transmembrane protein. It is termed P-gp for its association with the apparent permeability barrier to drugs that accompanies MDR. Most chemotherapeutic drugs are lipophilic and can enter cells passively. Drug-resistant cells exhibit a large elevation of P-gp expression in concert with the development of drug resistance. The P-gp serves as an energy-dependent pump to efflux cytotoxic drugs from the cell. This reduces the intracellular concentration of these drugs and maintains cell exposure to the drugs at a non-toxic level. At the biochemical level, it has been found that the P-gp pump is enormously catholic in its ability to bind and transport compounds. Recently, it has been reported that the P-gp pump can transport a wide variety of lipids (van Helvoort *et al.*, (1996) *Cell* 87:507).

2.1.1 MDR in the Treatment of Disease

MDR cells have the ability to decrease intracellular levels of chemotherapeutic drugs; the therapeutic effects of these drugs are then diminished. Furthermore, the cells become resistant not only to the drugs used in previous treatments, but also manifest resistance to other drugs. MDR represents a major obstacle to the successful treatment of such diseases as cancer, malaria, amoebic dysentery, and Leschmaniasis.

MDR is a common phenomenon in the treatment of cancers by chemotherapy. Tumors responding to a first chemotherapy often fail to respond to a second treatment with the same drug or with several chemically-unrelated drugs. Drug-resistant tumor cells overexpress the P-gp pump and can thus prevent cytotoxic chemotherapeutic agents, such vincristine and taxol, from reaching the intracellular levels needed to block cell division. This phenomenon has a negative impact on the success of cancer chemotherapy and patient survival. Resistance of malignant tumors to multiple chemotherapeutic agents is a major cause of treatment failure (Wittes *et al.*, (1986) *Cancer Treat. Rep.* 70:105).

The MDR phenotype is also detrimental to malarial therapy. Malaria is caused by infection by the protozoan, *Plasmodium* spp. It is characterized by paroxysms of chills, fever, and sweating, and by anaemia, splenomegaly, and a chronic relapsing course. The drug of choice for treating malaria is chloroquine. Unfortunately, in some malarious areas, such as Africa and South East Asia, malaria is rampant. Drug-resistant malarial plasmodia are able to keep the classic antimalarial agent, chloroquine, from reaching toxic levels in the plasmodial cells' food vacuoles by a mechanism involving the pfMDR transport protein. Control of resistant populations of malarial plasmodia in individual patients would benefit from synergists that interfere with the MDR mechanisms.

Members of the ABC superfamily of transport proteins have been implicated in MDR in other diseases as well. *Entamoeba hystolitica* is responsible for amoebic dysentery. Resistance of *Entamoeba hystolitica* to the clinically-used drug emetine is thought to involve a member of the

ABC superfamily (J. Samuelson *et al.*, (1990) *Molec. Biol. Parisitology* 38:281). *Leischmania* spp. is the causitive agent of the tropical disease Leschmaniasis. Clones of *Leischmania* spp. that show cross-resistance to several drug therapies display amplification of the genes for transport proteins of the ABC superfamily, whereas drug sensitive clones do not (Henderson *et al.*, (1992) *Molec. Cell Biol.* 12:2855).

2.1.2 MDR and the Blood Brain Barrier

It has been demonstrated that the P-gp also functions at the mammalian blood-brain barrier to protect the brain against circulating drugs (Schinkel *et al.*, (1994) *Cell* 77:491-502). In this work, a knockout mouse was created in which the gene coding for P-gp, mdr1a, was inactivated. The absence of P-gp rendered the mouse fatally susceptible to a topically applied insecticide, ivermectin, a P-gp substrate: without the blood-brain barrier P-gp, brain levels of ivermectin and sensitivity to ivermectin as a neurotoxin both increased by about two orders of magnitude. P-gp is also a component of the insect blood-brain barrier (Murray *et al.*, (1994) *J. Neurobiology* 25:23-34).

Neuroactive drugs, whether therapeutic, recreational, or insecticidal, including alkaloids and synthetic neurotoxins such as morphine, codeine, nicotine, and ivermectin, must cross the blood-brain barrier to act on central nervous system (CNS) targets. Many of these drugs are known or suspected substrates of P-gp For example, attempts to get chemotherapeutic drugs across the blood-brain barrier can be hampered by P-glycoprotein action at the barrier (Tamai and Tsuji (1996) *Advanced Drug Delivery Reviews* 19:401-424).

It is almost certain that CNS levels of neuroactive addictive compounds are reduced to some extent by P-gp at the blood-brain barrier. Insofar as P-gp at the blood-brain barrier acts to limit access of any of these compounds, it contributes to the phenomenon of drug tolerance. For example, to the extent that P-gp at the blood-brain barrier pumps brain nicotine out of the brain and back to the blood, higher peripheral nicotine is required by the addict brain to achieve the "desired" level. Research has shown that in specially adapted insects, an insect P-gp serves to excrete nicotine such

that a highly effective blood-brain barrier can be mounted against nicotine (Murray (1996) "A P-glycoprotein-like Mechanism in the Nicotine-Resistant Insect, *Manduca sexta*" PhD Thesis (University of Ottawa, ON, Canada); Murray et al. (1994) *J. Neurobiology* 25:23-34). There is also biochemical evidence that nicotine is a substrate for mammalian P-gp (Heather McDiarmid Honours Thesis, F Sharom's lab submitted to the Department of Chemistry and Biochemistry, University of Guelph, Sept 1995).

2.1.3 MDR and Pest Control

Pest control agents have long been used to control the undesired proliferation of pests, such as weeds, insects, and rodents. Unfortunately, approaches using these agents may fail due to MDR. For example, over 500 cases of insect resistance and/or multi-resistance to insecticides have been reported. Multi-resistance to insecticides is thought to arise primarily from the induction of cytochrome P-450 mediated polysubstrate monooxygenases (PSMOs). The PSMOs result in an increased biotransformation of the insecticide to a more polar and excretable product; consequently, there is reduced exposure of cellular targets in insects to toxic levels of the insecticide. Resistant insects can become resistant not only to the insecticide that triggered the enzyme induction, but also to a number of chemically-unrelated insecticides. Insecticide synergists that can prevent or inhibit specific detoxication enzymes are useful tools in the management of such cases (Raffa and Priester (1989) *J. Agric. Entomol.* 2:27-45).

Lignans and their derivatives have been observed to enhance the toxicity of insecticides by inhibiting the activity of the PSMOs; for example, in an insect microsomal preparation, dillapiol and biosynthetically-related lignans were shown to be effective inhibitors of PSM-based aldrin epoxidase activity (Bernard *et al.*, (1989) *Phytochem.* 28:1373).

MDR to pest control agents may also involve the ABC superfamily of transport proteins. P-gp is expressed to varying levels in many insect tissues, and its expression varies developmentally; for example, tobacco-feeding caterpillars have more blood-brain barrier P-gp than the non-feeding adult

form of the same insect. Pressure from applied pesticides could cause Darwinian selection of pesticide-resistant organisms that overexpress P-gp in any tissue and in any developmental stage, leading to MDR in those organisms. Insect Malpighian tubules have P-gp: they actively excrete a wide range of alkaloids, including nicotine and vinca alkaloids, and the P-gp inhibitor verapamil inhibits this excretion (Murray (1996) PhD Thesis, University of Ottawa, ON, Canada).

2.2 Strategies to Reverse MDR

Strategies designed to block expression of the MDR phenotype and to circumvent drug resistance are being sought. Reversal of the MDR phenotype can be accomplished by using compounds that are capable of blocking the function of members of the ABC superfamily of transport proteins, including P-gp and pfMDR. These compounds are called chemosensitizers, MDR inhibitors, or reversing agents. Particularly desirable are chemosensitizers that act at concentrations with little or no cytotoxic effect.

The chemosensitizers described to date may be grouped into six broad categories: calcium channel blockers; calmodulin antagonists; non-cytotoxic antracycline and Vinca alkaloid analogues; steroids and hormonal analogues; miscellaneous hydrophobic compounds; and cyclosporines. Most of these compounds are extremely lipophilic, and those in the first five groups are all heterocyclic, amphipathic substances (Ford *et al.*, (1990) *Pharmacol. Rev.* 42:155-199).

Verapamil, a calcium channel blocker, was the first described MDR modifier. Numerous investigators have described increases in accumulation of and decreases in resistance to natural product chemotherapeutic drugs in a number of different MDR cells treated with verapamil and other calcium channel blockers. Verapamil has been used with some success in multiple myeloma patients who no longer respond to salvage chemotherapy (Dalton *et al.*, (1989) *J. Clin. Oncol.* 7:415).

The mechanism by which verapamil, other calcium channel blockers, and calmodulin antagonists are thought to increase drug accumulation is by competing with anticancer drugs for binding to P-gp,

thereby inhibiting efflux of drug (Cornwell *et al.*, (1986) *J. Biol. Chem.* 261:7921-7928; Cornwell *et al.*, (1986) *J. Biol. Chem.* 262:2166-2170; Safa *et al.*, (1987) *J. Biol. Chem.* 7884-7888; and Akiyama *et al.*, (1988) *Mol. Pharmacol.* 33:144-147). The major problem associated with using verapamil to reverse MDR in patients is that it has dose-limiting cardiac toxicity due to blocking of the atrioventricular node. This toxicity prevents its use at concentrations required to reverse MDR *in vivo*.

A number of calmodulin antagonists have been found to be good MDR inhibitors *in vitro*. Trifluoperazine, a phenothiazine antipsychotic drug, has been noted to increase drug accumulation and decrease resistance in MDR cell lines (Tsuruo *et al.*, (1983) *Cancer Res.* 43:2905-2910; Ford *et al.*, (1989) *Mol. Pharmacol.* 35:105-115). Neurotoxicity of this compound, however, is dose-limiting.

Cyclosporins are immunosuppressive agents which can also potentiate toxicity of anticancer agents at clinically achievable concentrations. In some cell lines, they appear to enhance toxicity and increase drug accumulation in sensitive and resistant cells (Chambers *et al.* (1989) *Cancer Res.* 49:6275-6279). Cyclosporin A competitively inhibits drugs such as vincristine and vinblastine from binding to the P-gp (Tamai *et al.*, (1990) *J. Biol. Chem.* 265:16509-16513). The use of cyclosporin A to modulate drug resistance may be hampered by irreversible nephrotoxicity and immunosuppression in patients already compromised by myelosuppressive chemotherapy. The use of non-immunosuppressive cyclosporin analogues may be less toxic.

Hormone analogues, such as the antiestrogens tamoxifen and toremifene, are employed in the chemotherapy of breast cancers. These compounds can also modulate resistance of estrogen receptor-negative MDR cell lines via estrogen receptor independent mechanisms (Ramu *et al.*, (1984) *Cancer Res.* 44:4392-4395; and Bermin *et al.*, (1991) *Blood* 77:818-825).

Some compounds act as chemosensitizers by interfering with the energy required to pump cytotoxic

drugs out of the cell. These include 2,4-dinitrophenol, 2,4-dinitrocresol, and hydrogen cyanide. Most compounds that interfere with the energy required to pump cytotoxic drugs out of cells are toxic to normal cells; thus, they are not ideal for clinical use.

Chemosensitizers have also been used in the treatment of MDR in malaria. It has been shown that the treatment of monkeys infected with chloroquine-resistant malaria parasites is improved in the presence of a chemosensitizer (Juranka *et al.*, (1989) *FASEB J.* 3:2583).

The identification of several pharmacologic agents that can reverse the MDR phenotype *in vitro* has not, to date, identified MDR inhibitors with good clinical efficacy. This is primarily due to the fact that *in vivo* concentrations necessary to reverse MDR cannot be achieved without substantial toxicity to patients. For this reason, there remains a need for an effective clinical method of treating MDR.

2.3 Collateral Sensitivity

An alternative strategy involves developing chemosensitizers that are specifically cytotoxic to MDR cells, leaving normal cells unaffected. The development of MDR is often accompanied by an increased sensitivity to membrane-active agents; this phenomenon is known as collateral sensitivity. It has been reported that collateral sensitivity occurs in MDR cells with the use of membrane active agents including detergents, anaesthetics, and steroids (Bech-Hansen *et al.*,(1976) *J. Cell. Physiol.*, 88:23-32; Riordan and Ling (1985) *Pharmol. Ther.* 28:51-75; Loe and Sharom (1993) *Br. J. Cancer* 68:342-351). As well, collateral sensitivity to drugs has been reported with transfectants (Juranka *et al.*, (1989) *FASEB J.* 3:2583-2592). Unfortunately, many detergents are poor candidates for therapeutic use because of their undesirable non-target effects, especially on membranes.

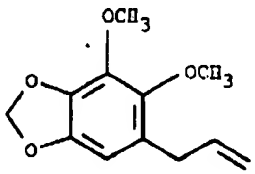
Some lignans have been reported to enhance cytotoxicity with cultured MDR cells (Pezzutto (1993) *J. Nat. Prod.* 56: 233-239). For example, phyllanthin, an oxodiarylbutane, has recently been found to be more toxic to an MDR cell line than to its drug-sensitive parental cell line (Somanabandhu *et al.*, (1993) *J. Natl. Prod.*, 56(2):233-239).

Several chemosensitizers, including calmodulin inhibitors (eg. trifluoperazine), calcium channel blockers (eg. verapamil, bepridil), and other classes of non-related compounds, have also been reported to be more toxic to MDR cells lines than to their parental cell lines (Schuurhuis *et al.*, (1990) *Int. J. Cancer* 46:330-336). Despite these findings, the clinical use of these chemosensitizers is questionable due to their dose-limiting toxicity.

This review of chemosensitizers currently available indicates that although these compounds are effective in reversing the MDR phenotype *in vitro*, the concentrations necessary to produce this result limit their use *in vivo*; thus, a need remains for a clinically acceptable method of treating MDR.

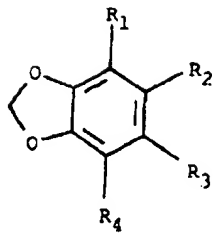
2.4 Dillapiol Compounds

Dillapiol is a monolignol (phenylpropanoid derivative) that is produced biosynthetically via the Shikimic acid pathway. It has been reported that dillapiol is synthesized in only a few species of higher plants and is not currently available commercially. The structure of dillapiol is depicted in Formula I.



Formula I

In addition to the core structure of dillapiol, a number of analogues and derivatives exist (hereinafter referred to as dillapiol compounds), represented, for example by the following Formulae (II) and (III):

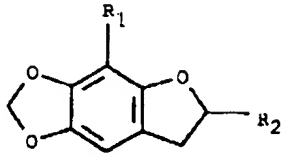


Formula II

wherein,

R_1 is selected from H and OCH_3; and

R_2 is selected from CH_3;



Formula III

wherein,

R_1 is selected from H, OH, and OCH_3;

R_2 is selected from H, OCH_3, and $CH_2CH_2CH_3$;

R_3 is selected from H, OCH_3, $CH_2CH{=}CH_2$, $CH{=}CHCH_3$, $CH_2CH_2CH_3$, and $CH_2OCH_2CH_2OCH_2CH_2OC_4H_9$; and

R_4 is selected from H, $CH_2CH{=}CH_2$, and OCH_3.

Where R_1 is OCH_3, R_2 is OCH_3, R_3 is H, and R_4 is $CH_2CH{=}CH_2$, the compound may be called pseudodillapiol. Where R_1 is OCH_3, R_2 is H, R_3 is $CH_2CH{=}CH_2$, and R_4 is OCH_3, the compound may be called apiol. Where R_1 is OCH_3, R_2 is OCH_3, R_3 is $CH{=}CHCH_3$, and R_4 is H, the compound may be called isodillapiol. Where R_1 is OCH_3, R_2 is OCH_3, R_3 is $CH_2CH_2CH_3$, and R_4 is H, the compound may be called dihydrodillapiol. Where R_1 is H, R_2 is $CH_2CH_2CH_3$, R_3 is $CH_2OCH_2CH_2OCH_2CH_2OC_4H_9$, and R_4 is H, the compound may be called piperonyl butoxide.

Dillapiol is a major constituent (27%) of Indian dill (*Anethum sowa* Roxb.) seed oil. A method of extracting dillapiol from commercial dill seed oil has been reported (Tomar *et al.*, (1979) *J. Agr. Food Chem.* 27:547). Dillapiol has also been isolated from the dried leaves of *Piper aduncum L.* (Orjala *et al.*, (1993) *Planta Medica* 59:546-551). Methods for synthesizing dillapiol are also known in the art (Baker and Subrahmanyan (1934) *J. Chem. Soc.* 1681; Dallacker (1969) *Chem. Berichte* 102:2663-2668; Cannon *et al.*, (1980) *J. Sci. Soc. Thailand* 6:59).

The natural isolate pseudodillapiol has been extracted from the Jamaican *Piper aduncum* and *Piper hispidum* (Burke and Nair (1986) *Phytochemistry* 25:1427-1430). Methods of synthesizing dillapiol compounds have also been reported in the literature (U.S. Patent Nos. 4,876,277 and 4,803,290; Muraleedharan and Burke (1990) *J. Agric. Food Chem.* 38:1093-1096; Talwar *et al.*, (1966) *Indian Perfumer* 10:43; Saxena *et al.*, (1977) *Pyrethrum Post* 14:41; Tomar *et al.*, (1979) *J. Agr. Food Chem.* 27:547; Diamician and Sliber (1896) *Berichte* 29:1799; Tomar *et al.*, (1979) *Agric. Biol. Chem.* 43:1479-1483; Dallacker (1969) *Chem. Berichte* 102:2663-2668).

There have been reports on the use of dillapiol as a synergist with insecticides; dillapiol compounds have been used for the management of horticultural, silvicultural, and agricultural pests. These compounds, including piperonyl butoxide, have been shown to have synergistic activity with pyrethrum insecticides (Nair *et al.*, (1985) *Agric. Biol. Chem.* 50:3053-3058; Devakumar *et al.*, (1985) *Agric. Biol. Chem.* 49:725-728; Tomar *et al.*, (1979) *Agric. Biol. Chem.* 43:1479-1483; Indian Patent No. 128,129 (1969)). Dillapiol has also been shown to have synergistic activity for N-methyl carbamates (Lichtenstein *et al.*, (1974) *J. Agr. Food Chem.* 22:658; Tomar *et al.*, (1978) *Indian J. Ent.* 40:113). In this regard, dillapiol has been shown to function by inhibiting polysubstrate mono-oxygenases (PSMOs) (Bernard *et al.*, (1989) *Phytochem.* 28:1373).

Monomers of lignans, such as apiol, dillapiol, and safrol, all contain a methylenedioxyphenyl (MDP) ring (Casida *et al.*, (1970) *J. Agr. Food Chem.* 12:24-25). This MDP ring is thought to be responsible in part for the ability of these monomers to act as potent synergists of several classes of insecticides including pyrethrins, carbamates and organophosphates (Lichtenstein *et al.*, (1974) *J. Agr. Food Chem.* 22:658-664; Mukerjee *et al.*, (1979) *J. Agr. Food Chem,* 27(6):1209-1211). The sesamine-type synergists and their synthetic derivatives act by interfering with the rate of detoxification of the insecticide (Wilkinson and Hickes (1969) *J. Agr. Food Chem.* 17:829-836). This interference, due in part to the presence of the MDP ring, is caused by preferential binding of the compounds to the active sites of the main detoxification enzymes in insects, the cytochrome P-450 dependent PSMOs (Ahmad *et al.* in: L.B. Brattsten and S. Ahmed (eds), *Molecular Aspects of Insect-Plant Associations* (New York: Plenum Press, 1986) 73-127).

Dillapiol compounds have also been shown to be useful as antimicrobial agents and, more specifically, as antifungal and antibacterial agents (U.S. Patent Nos. 4,876,277 and 4,803,290; Nair and Burke (1990) *J. Agric. Food Chem.* 38:1093-1096).

This background information indicates that there remains a need for an effective method of treating

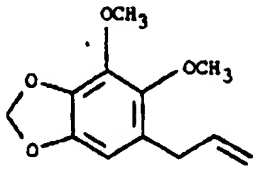
MDR in medicine and in pest control management.

This background information is provided for the purpose of making known information believed by the applicant to be of relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Publications referred to throughout the specification are hereby incorporated by reference in their entireties into this application.

3. SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a method of reducing MDR activity and/or killing cells expressing an MDR phenotype.

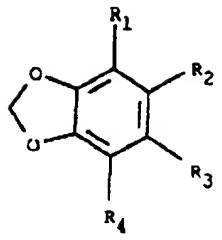
The present invention describes a method of using dillapiol or its analogues and derivatives to affect cells that express MDR activity. The structure of dillapiol is depicted in Formula I.



Formula I

In addition to the core structure of dillapiol, a class of dillapiol compounds is included as the method of this invention comprising analogues and derivatives, represented, for example by the following

Formulae (II and III):

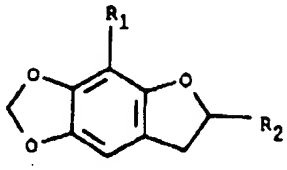


Formula II

wherein

R_1 is selected from H and OR, wherein R is selected from C_1-C_8 alkyl, alkenyl, and alkynyl groups; and

R_2 is selected from CH_3 and CH_2Br, and



Formula III

wherein R_1, R_2, R_3, and R_4 may be the same or different and are selected from the following:

R_1	R_2	R_3	R_4
H	H	H	H
R_5	R_5	R_5	R_5
OH	OH	OH	OR_5
OR_5	OR_5	OR_5	
R_5OH		R_5OH	
R_5OR_5		R_5OR_5	
CHO		$CH(OR_5)R_5$	
OCHO		$CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2CH_3$	
$CH(OR_6)CH_2CH_3$		$CH(OR_6)CH_2CH_3$	
$CH_2CH(OR_6)CH_3$		$CH_2CH(OR_6)CH_3$	
$CH_2CH_2CH_2OR_6$		$CH_2CH_2CH_2OR_6$	
$CH[OC(O)R_6]CH_2CH_3$		$CH[OC(O)R_6]CH_2CH_3$	
$CH_2CH[OC(O)R_6]CH_3$		$CH_2CH[OC(O)R_6]CH_3$	
$CH_2CH_2CH_2OC(O)R_6$		$CH_2CH_2CH_2OC(O)R_6$	
$C(O)R_6$		CHCHCH$_2$C(OH)PhPh	
$CH{=}CHCOOCH_3$			
CH_2COCH_2X			
$CH_2OCH_2C(O)OCH_2CH_3$			

wherein:

R_5 is selected from H and C_1-C_8 alkyl, alkenyl, and alkynyl groups;

R_6 is selected from R_5, phenyl groups, and $C_6H_4(OCH_3)_n$ where n=1,2, or 3; and

X is a halogen.

It is to be understood that newly developed (synthesized or isolated) members of the class of

compounds of which dillapiol is a representative member are considered to be within the scope of this invention. Moreover, derivatives such as halogenated or otherwise substituted derivatives based on the dillapiol class of compounds are also considered to be within the scope of this invention. It is also to be understood that those members which demonstrate biological activity in terms of their ability to exert a negative effect on MDR are the compounds for use in the method of this invention.

In one embodiment, the present invention is used to treat pathologies and physiologies in which MDR plays a role. In a specific embodiment, the present invention is used alone to adversely affect MDR cells involved in pathologies and physiologies such as cancer and malaria.

In another specific embodiment, the present invention is used in conjunction with other chemical agents to treat pathologies and physiologies in which MDR plays a role. Dillapiol or its analogues and derivatives increase the net accumulation of these agents in MDR cells and thus increase their efficacy. In a particular embodiment, this method is applied to reduce MDR activity in cancerous cells in order to restore the efficacy of chemotherapy. In another particular embodiment, this method is applied to cells, microbes, and pathogens that have become MDR, such as the malaria-causing parasite *Plasmodium* spp., *Leischmania* spp. responsible for Leschmaniasis, and the causitive agent of amoebic dysentery *Entamoeba* spp. In yet another particular embodiment, this method is used to inhibit the transport of drugs across the blood-brain barrier in order to assist in therapeutic treatments.

In yet another embodiment, the present invention relates to a method to augment addiction and tolerance therapies, comprising administering dillapiol or its analogues and derivatives in conjunction with other chemical agents. In a particular embodiment, this method is used in a smoking cessation therapy to maximize the amount of nicotine that remains within the CNS.

In a further embodiment, the present invention relates to a method of reducing MDR to pest control agents, comprising administering dillapiol or its analogues and derivatives either alone or in conjunction with other chemical agents. In particular, this method is applied to improve the efficacy

of insecticides.

In another embodiment, the present invention is used *in vitro* to identify chemical compounds that increase in effectiveness in the presence of dillapiol compounds.

In another embodiment, the present invention is used *in vitro* to tailor therapies to individual patients.

In yet another embodiment, the present invention is used in conjunction with other chemosensitizers to enhance their effect.

Various other objects and advantages of the present invention will become apparent from the detailed description of the invention.

4. BRIEF DESCRIPTION OF THE DRAWINGS

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following preferred embodiments of the invention taken in connection with the accompanying drawings in which:

Figure 1 depicts differential uptake of ^{3}H-vinblastine by drug-sensitive and MDR cells. In this figure, panel A shows the results obtained with hamster cells, wherein the abbreviations are as follows: AUXB1 indicates parental drug-sensitive cells; C5 indicates MDR cells; and B30 indicates MDR cells. Panel B shows murine cells, wherein NIH shows the results of parental drug-sensitive cells and G185 depicts the results of MDR cells. Data represent the mean of two (C5) or four (all other cell types) experiments.

Figure 2 demonstrates differential uptake of ^{3}H-vinblastine by the Chinese hamster ovary MDR-selected cells B30 and their drug-sensitive parental cells AUXB1 in the presence of the P-glycoprotein inhibitor verapamil (160 μM), or various concentrations of dillapiol.

Figure 3 shows differential uptake of ^{3}H-vinblastine by the murine fibroblast cells transfected with a human MDR gene G185 and their drug-sensitive parental cells NIH after 60 minutes of incubation with dillapiol and selected dillapiol derivatives. The abbreviations are as following: DIL is dillapiol, 65 nM; der1 is derivative 1, 150 nM; der2 is derivative 2, 150 nM; der3 is derivative 3, 150 nM; der4 is derivative 4, 150 nM; der5 is derivative 5, 150 nM; and PBO is piperonyl butoxide, 65 nM. Uptake in control was 53,000 + 2,000 dpm and 19,000 ± 1,500 dpm/10^6 cells for NIH and G185, respectively.

Figure 4 shows differential uptake of ^{3}H-vinblastine by the Chinese hamster ovary MDR selected cells B30 and their parental cells AUXB1, and by the murine fibroblast cells transfected with a human MDR gene G185 and their parental cells NIH, after 60 minutes of incubation with 65 nM of dillapiol (DIL), 160 μL of verapamil (VER), and 65 nM of piperonyl butoxide (PBO).

Figure 5 shows the effects of dillapiol on the accumulation of α-terthienyl in the tissues of 4th instar mosquito larvae, *Aedes atropalpus* (Diptera: Culicidae). Mosquito larvae were incubated in water with α-terthienyl (50 ppb) and dillapiol for 24 hours in darkness.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1 Definitions

The following common abbreviations are used throughout the specification and in the claims:

The term "analogue" means a chemical compound having a structure similar to that of another compound but differing from it in respect to a certain component; thus, a dillapiol analogue is a chemical compound with a structure similar to that of dillapiol.

The term "chemosensitizer" means a compound that allows an increase in the net accumulation of toxic compounds in MDR cells.

The phrase "concurrently administering" means that a chemical agent and a dillapiol compound are administered either (a) simultaneously, or (b) at different times during the course of a common treatment schedule. In the latter case, the two compounds are administered at times sufficiently close for the dillapiol compound to enhance the action of the chemical agent. This may be within one month, one week, one day, one hour, or one minute.

The term "derivative" means any chemical compound derived from, or regarded as being derived from, another compound either directly or by modification or partial substitution; thus, a dillapiol derivative is a chemical compound that either was, is, or can be regarded as having been derived from dillapiol. For example, compounds such as halogenated compounds that can be considered as derived from a member of the dillapiol class of compounds are considered within the scope of this invention.

The term "dillapiol compound" includes dillapiol and its analogues and derivatives. The members of the dillapiol class of compounds that are considered to be part of the method of this invention are members of this class that are selected on the basis of their ability to exert a negative effect on MDR

The term "net accumulation" describes the net consequence of influx and efflux of drugs, so that at a mechanistic level increasing net accumulation can occur from enhanced influx, from inhibiting efflux, or from both.

The term "MDR" is an abbreviation for multidrug resistance. Multidrug resistance is the state in

which cells are resistant to a variety of chemical agents. This resistance is characterized in part by decreased intracellular chemical retention of the agents as a result of their increased efflux. This increased efflux is mediated by the overexpression of members of the ATP-binding cassette superfamily of transport proteins. As used herein, multidrug resistance includes multi-resistance to pest control agents.

The term "MDR cells" as used herein is used to denote cells expressing an MDR phenotype or exhibiting MDR.

The term "pest control agent" indicates a substance that serves to repel a pest from a living organism, or decrease or inhibit the growth, development, or destructive activity of a pest. A pest can be a plant, an animal, or a microorganism. Exemplary pests include insects, spiders, nematodes, fungi, weeds, bacteria, and other microorganisms; thus, pest control agents include insecticides, pesticides, fungicides, herbicides, nematicides, acaricide, molluscicide, antiparisitic agents, antibiotics, and anti-microbials. A pest control agent can also be a mixture of two or more agents. Pest control agents are commercially available. An exemplary list of such substances can be found in U.S. Patent No. 4,911,952, the disclosure of which is incorporated herein by reference.

5.2 General Description of the Invention

The present invention resides in the discovery that the natural monolignol dillapiol and its analogues and derivatives affect cells expressing MDR activity. The method of this invention entails the use of dillapiol compounds to affect cells expressing MDR activity. In one embodiment, dillapiol compounds can be used alone in order to exert a direct toxic effect on cells presenting an MDR phenotype. In another embodiment, dillapiol compounds can be used as chemosensitizers to chemical agents, including drugs such as chemotherapeutic agents, insecticides, and addictive substances such as morphine and nicotine, in order to increase the net accumulation of these agents in MDR cells. This method is useful for research, for determining effective therapeutic combinations, for treating

pathologies and physiologies in which MDR plays a role such as certain cancers and diseases (eg. malaria), for treating states of addiction and tolerance (eg. nicotine and opiate addiction), for pest control maintenance, and for enhancing the effects of other chemosensitizers.

5.3 Use of Invention to Treat Pathologies and Physiologies in which MDR Plays a Role

5.3.1 Use of Invention Alone

In one embodiment, the method of the present invention is used alone to adversely affect MDR cells. Because dillapiol compounds are selectively toxic to MDR cells, they can be used to kill MDR cells at concentrations that are not toxic to other cells. This embodiment provides a method of treating a subject comprising administering to the subject a therapeutially effective amount of a compound of formula (I), (II), or (III) above.

Subjects to be treated by the method of the present invention include animals and humans; preferably, subjects are mammalian.

In a specific embodiment, dillapiol compounds can be administered to cancer patients as a means of treating cancer cells which have become MDR in the course of therapy.

In another specific embodiment, the method is used to treat malaria.

5.3.2 Use of Invention in Conjunction with other Chemical Agents

In another embodiment, the method of the present invention is used in conjunction with therapeutic agents to affect MDR cells. Because of the general efficacy of dillapiol compounds in increasing the net accumulation of therapeutic agents in MDR cells, these compounds can be used to increase the efficacy and/or reduce the total administered dose of all types of therapeutic agents used in human or veterinary medicine.

This embodiment provides a method of treating a subject comprising administering to the subject an effective amount of a compound of formula (I), (II), or (III) above in combination with one or more therapeutic agents. The pharmaceutical compositions and administration can be as described below. The therapeutic agents to be employed in combination with the dillapiol compounds can be used in therapeutic amounts as indicated in the current version of the Physicians' Desk Reference, or such therapeutically useful amounts as would be known to one of ordinary skill in the art.

When administered as a combination, the therapeutic agents can be formulated as separate compositions, which are given either at the same time or at different times, or as a single composition.

Subjects to be treated by the method of the present invention include both humans and animals, and are preferably mammalian.

5.3.2.1 To Treat Cancer

In a specific embodiment, dillapiol compounds can be administered to cancer patients in conjunction with a cancer chemotherapeutic agent as a means of treating cancer cells that have become MDR in the course of therapy. Dillapiol compounds increase the net accumulation of cancer chemotherapy agents in MDR cells. In this manner, dillapiol compounds can be used to restore, completely or partially, the efficacy of cancer chemotherapy agents in MDR cells.

Cancer chemotherapeutic agents can be administered with the dillapiol compounds. The coadministeration is designed to increase net accumulation of the chemotherapeutic agent following reversal of the MDR phenotype, causing the agent to accumulate in amounts effective for cytotoxicity to cancer cells. Any cancer chemotherapeutic agent can be administered according to the present invention. Examples of cancer chemotherapy agents that may be used in combination with the dillapiol compounds are Vinca alkaloids such as vincristine, vinblastine, anhydrovinblastine, and vindesine; epipodophyllotoxins such as etoposide and teniposide; antibiotics; anthracycline antibiotics; actinomycin D; puromycin; gramicidin D; taxol; taxotere; colchicine; topoisomerase I and II

inhibitors; cytochalasin B; emetine; maytansine; and amsacrine.

In order to interfere with MDR in cancer cells, dillapiol compounds need be administered only in amounts sufficient to interfere with MDR. Dillapiol compounds may be administered first; the chemotherapeutic drug may then be administered in a dose lethal to the cancer cells. Alternatively, the cancer chemotherapeutic drug may be administered simultaneously with the dillapiol compound.

Exemplary of cancer cells that express P-gp and are intrinsically resistant are adenocarcinoma cells, pancreatic tumor cells, pheochromocytoma cells, carcinoid tumor cells, chronic cyelogenous leukemia cells in blast crisis, renal cells, hepatocellular tumor cells, adrenal cancer cells, and colon cancer cells Exemplary of cancer cells having the ability to become MDR following chemotherapy are neuroblastoma cells, adult acute lymphocytic leukemia cells, adult acute nonlymphocytic leukemia cells, nodular poorly differentiated lymphoma cells, breast cancer cells, and ovarian cancer cells: Other cancer cells may also be treated by the methods of this invention.

5.3.2.2 To Treat Diseases Caused by Parasites and Pathogens

In another particular embodiment, the method of the present invention is used to restore sensitivity to therapeutic agents in parasitic organisms and in disease-causing microbial pathogens expressing the MDR phenotype. According to the method of the present invention, therapeutic agents are administered in conjunction with the dillapiol compounds. The co-administration is designed to increase net accumulation of the therapeutic agent such that the agent is present in amounts effective for cytotoxicity to the parasitic organisms or microbial pathogens. Any therapeutic agent can be administered according to the present invention. For example, the method of the present invention could be used in conjuction with avermectin for *Ankylostoma duodenalis*, with vancomycin for bacterial infections, with micranasole nitrate for fungal infections including *Candida albicans*, with Suramin for river blindness caused by *Onchocerca volvulus*, and with Nifurtimox for African and American trypanosomiasis.

Dillapiol compounds are particularly useful in malarial therapy, where they inhibit the pfMDR pump of *Plasmodium* spp. In a specific embodiment, the method of the present invention comprises administering dillapiol compounds in conjunction with antimalarial agents such as quinine, chloroquine, mefloquine, and artemisinin (artesunate). In one example, dillapiol would be combined with the antimalarial agent in a weight ratio of 5:1, but the ratio could vary from 0.1:1 to 100:1.

In another specific embodiment, the method of the present invention is used to restore drug sensitivity in *Entamoeba* spp. and *Leischmania* spp., comprising adminstering dillapiol compounds in conjunction with therapeutic agents such as emetine or metronidasole for *Entamoeba histolytica* and sodium stibogluconate for *Leischmaniasis* spp.

5.3.2.3 To Treat CNS Drug Tolerance

In another specific embodiment, the method of the present invention is used to combat the phenomenon of CNS drug tolerance. P-gp-mediated extrusion of compounds from the brain reduces their efficacy. Dillapiol compounds increase the net accumulation of drugs and chemicals in the brain through their ability to inhibit P-gp at the blood-brain barrier; thus, dillapiol compounds can be used in conjunction with drugs and chemicals targeted to the CNS. Dillapiol compounds allow smaller quantities of circulating drugs to achieve the desired CNS effect, thus reducing the required dose and any potential peripheral side effects of the administered drug. Examples of CNS drugs that could be used in conjunction with dillapiol compounds are headache drugs such as G_p-blockers, sertonin modulators, and ergots; antipsychotics such as haloperidol; antidepressants with anticholinergic side effects such as amitryptilene and doxepin; and antiparkinsonian drugs such as leva-dopa. As another example, dillapiol compounds can be useful clinically in combatting blood-brain barrier extrusion of calcium channel blockers such as dihydropyridines used to alleviate excitotoxicity in the case of stroke.

5.3.2.4 To Treat Addiction and Tolerance

In another embodiment, the method of the present invention can be used to treat addiction and

tolerance. In a specific embodiment, dillapiol compounds can be taken concurrently with addictive substances during treatment for addiction to these substances. For example, dillapiol compounds can be taken concurrently with nicotine products, such as nicotine-containing chewing gums, nicotine patches, or other slow-release devices, administered in smoking cessation therapies. The dillapiol compounds allow smaller quantities of circulating nicotine to achieve the desired CNS effect, thus reducing the required dose and any potential side effects of the administered nicotine.

5.4 Use of the Invention in Pest Control Management

Dillapiol compounds are also able to enhance the uptake of pest control agents, such as insecticides, pesticides, fungicides, herbicides, nematicides, acaricides, molluscicides, antiparasitic agents, antibiotics, and anti-microbials in target cells. Dillapiol compounds can be used to counteract the protective effect of P-gp-mediated drug transport at any site, including gut, blood-brain barrier, or Malpighian tubule, in insects or other pests. Dillapiol's collateral sensitivity action would kill or generally disable MDR cells in the organism and thus disable MDR pests as they arise in a population. This is a different mode of action from the known ability of dillapiol to inhibit PSMOs; thus, this is a novel method for maximizing the efficacy of pest control treatments.

5.4.1 Use of Invention Alone

In one embodiment, the method of the present invention is used alone to adversely affect MDR cells. Because dillapiol compounds are selectively toxic to MDR cells, they can be used to kill MDR cells at concentrations that are not toxic to other cells. This embodiment provides a method of treating a subject comprising administering to the pest an effective amount of a compound of formula (I), (II), or (III) above.

As a specific example, the tobacco hornworm overexpresses the P-gp in certain tissues such as the neuropile and Malpighian tubules; this allows it to avoid the accumulation and toxic effects of the natural insecticide nicotine present in its diet (Murray *et al.*, (1994) *supra*). Dillapiol compounds

could be administered to the tobacco hornworm to inhibit the P-gp pump, allowing nicotine to accumulate until it reaches toxic levels.

5.4.2 Use of Invention in Conjunction with other Chemical Agents

In another embodiment, the method of the present invention is used in conjunction with pest control agents to affect MDR cells for pest control. For example, dillapiol compounds could be used to increase the accumulation and efficacy of natural and synthetic insecticides by inhibiting the P-gp pump in insects. Examples of insectides include pyethroids, carbamates, organophosphates, plant-derived antifeedants such as azadirachtin from neem and its derivatives, alpha terthienyl and its derivatives, gedunin, and insecticidal extracts of other species of the family *Meliaceae*. Examples of insect pests that could be treated by the method of the present invention include *Spodoptera frugiperda*, *Drosophila*, and the mite *T. urtica*, all of which express the P-gp pump.

5.5 Use of Invention in Research

In one embodiment, the method of the present invention relates to the use of dillapiol compounds *in vitro* to identify chemical compounds that increase in effectiveness in the presence of dillapiol compounds. MDR cells *in vitro* can be exposed to a potential compound in the presence of dillapiol compounds. The potential compound could be labeled, using such labels as radioisotopes, or it could be cold.

Dillapiol compounds could be used in standard drug uptake experiments. MDR cells *in vitro* could be exposed to radioactive compounds of interest in conjunction with dillapiol compounds to determine if the uptake of the test compound increases into a therapeutically useful range in the presence of dillapiol compounds.

Dillapiol compounds could also be used in toxicity studies. MDR cells *in vitro* could be exposed to test compounds in conjunction with dillapiol compounds to determine whether the test compounds increase in cytotoxicity in the presence of dillapiol compounds.

Possible test compounds include cytotoxic drugs, cancer chemotherapies, therapeutic agents, and pest control agents.

5.6 Use of Invention to Assess Drug Therapies

Dillapiol compounds can also be used *in vitro* to tailor drug therapies to individual patients. A tissue sample could be taken from the patient and used *in vitro* to assess the efficacy of a particular therapeutic protocol in combination with dillapiol compounds. As an example, a biopsy could be taken from a tumor, the cells grown in culture, and various combinations of dillapiol compounds and known or potential chemotherapeutic agents tested for their ability to affect the MDR tumor cells. This regimen allows for determination of effective combinations for chemotherapy by demonstrating which chemotherapeutic drugs can be effectively accumulated in MDR cells as a result of the addition of dillapiol compounds.

5.7 Use of Invention to Enhance the Effect of other Chemosensitizers

Dillapiol compounds can be used to enhance the effect of other chemosensitizers. In this embodiment, the dillapiol compounds are added to a regimen that already includes the use of a chemosensitizer being co-administered with a chemotherapeutic agent whose intracellular accumulation in MDR cells is desired. Dillapiol compounds should be administered concurrently with another known chemosensitizer to enhance the cytotoxicity or reversing properties of the chemosensitizer.

5.8 Compositions

5.8.1 Pharmaceutical Compositions

The present invention involves pharmaceutical compositions containing dillapiol compounds in combination with one or more pharmaceutically acceptable, inert or physiologically active, diluents or adjuvants. The compounds of the invention can be freeze dried and, if desired, combined with other pharmaceutically acceptable excipients to prepare formulations for administration. These

compositions may be presented in any form appropriate for the administration route envisaged.

Dillapiol compounds may be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, or intrasternal injections, or infusion techniques.

A pharmaceutical composition may be prepared comprising dillapiol compounds and a pharmaceutically acceptable carrier. One or more dillapiol compounds may be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants and if desired other active ingredients. The pharmaceutical compositions containing dillapiol compounds may be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion hard or soft capsules, syrups, or elixirs.

Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions. Such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavouring agents, colouring agents, and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in an admixture with non-toxic pharmaceutically acceptable excipients suitable for the manufacture of tablets. These excipients include inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate, or sodium phosphate; granulating and disintegrating agents, such as corn starch or alginic acid; binding agents, such as starch, gelatin, or acacia; and lubricating agents, such as magnesium stearate, stearic acid, or talc. The tablets may be uncoated or they may be coated by techniques known to delay disintegration and absorption in the gastrointestinal tract, thereby providing a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed.

Compositions for oral use may also be presented as hard gelatin capsules wherein the active

ingredient is mixed with an inert solid diluent; for example, calcium carbonate, calcium phosphate, or kaolin may be used. Alternatively, compositions for oral use may also be presented as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium.

Aqueous suspensions contain active materials in an admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, such as sodium carboxymethylcellulose, methyl cellulose, hydropropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth, and gum acacia. Dispersing or wetting agents may include the following: naturally-occurring phosphatides, such as lecithin; condensation products of an alkylene oxide with fatty acids, such as polyoxyethylene stearate; condensation products of ethylene oxide with long chain aliphatic alcohols, such as hepta-decaethyleneoxycetanol; condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol, such as polyoxyethylene sorbitol monooleate; or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, such as polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, such as ethyl or n-propyl p-hydroxy-benzoate, one or more colouring agents, one or more flavouring agents, or one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions may be formulated by suspending the active ingredients in a vegetable oil such as arachis oil, olive oil, sesame oil, or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, such as beeswax, hard paraffin, or cetyl alcohol Sweetening agents, such as those set forth above, and flavouring agents may be added to provide palatable oral preparations. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

Pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oils phase may be a vegetable oil, such as olive oil or arachis oil, or a mineral oil, such as liquid paraffin, or mixtures of these. Suitable emulsifying agents may be naturally-occurring gums, such as

gum acacia or gum tragacanth, naturally-occurring phosphatides, such as soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, such as sorbitan monoleate, and condensation products of the said partial esters with ethylene oxide, such as polyoxyethylene sorbitan monoleate. The emulsions may also contain sweetening and flavouring agents. Syrups and elixirs may be formulated with sweetening agents, such as glycerol, propylene glycol, sorbitol, or sucrose. Such compositions may also contain a demulcent, a preservative, and flavouring and colouring agents.

The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a solution or suspension in a non-toxic parentally- acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Dillapiol compounds may also be administered in the form of suppositories for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature; thus, it will melt in the rectum to release the drug. Such materials are cocoa butter and polyethylene glycols.

Dillapiol compounds can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to a compound of the present invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the

phospholipids and phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art (See: Prescott (ed.) *Methods in Cell Biology*, Volume XIV (New York, Academic Press, 1976) at 33).

Dillapiol compounds may be administered parenterally in sterile medium. The compound, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anaesthetics, preservatives, and buffering agents can be dissolved in the vehicle.

For the compounds of this invention, the dose to be administered, whether a single, multiple, or daily dose, will vary with the particular compound being used. Factors to consider when deciding upon a dose regimen include potency of the compound, route of administration, age, body weight, and general health of the recipient, and the severity of the particular condition undergoing therapy. The dosage to be administered is not subject to defined limits, but will usually be an effective amount

5.8.2 Pest Control Compositions

When pest control is involved, subjects to be treated by the method of the present invention are pests, including plants, animals, and microorganisms.

The pest control compositions are prepared in manners known to one skilled in the art. A pest control composition will commonly contain an active ingredient or pest control agent, a wetting agent such as a surfactant, and a solvent such as water. Optionally, an oil such as conventional crop oil, can also be included as a solvent for emulsion concentrates. The dillapiol compounds may be mixed with pest control agents and conventional inert agronomically or physiologically acceptable (i.e. plant and mammal compatible and/or insecticidally inert) diluents or extenders usable in conventional compositions as is well known in the art. If desired, adjuvants such as surfactants, stabilizers, antifoam agents, and antidrift agents may also be added. Examples of pest control compositions include aqueous or other agronomically acceptable suspensions and dispersions, oily

dispersions, pastes, dusting powders, wettable powders, emulsifiable concentrates, flowables, granules, baits, invert emulsions, aerosol compositions, and fumigating candles.

Adhesives, such as carboxymethylcellulose and natural and synthetic polymers in the form of powders, granules, or lattices, such as gum arabic, polyvinyl alcohol, polyvinyl cellulose, and polyvinyl acetate, can be used in the pest control compositions to improve the adherence of the pest control agent. Furthermore, a lubricant such as calcium stearate or magnesium stearate may be added to a wettable powder or to a mixture to be granulated.

Pest control compositions suitable for use with dillapiol compounds of the present invention include both package and tank mix compositions.

The components employed in the pest control composition can be combined in any order. For example, the composition can be prepared by starting with a pest control agent and adding the various components in any order. Water can be employed, if desired. The amount of water employed to prepare the concentrate or final application concentration, as in a spray, is adjusted as necessary. The concentrate and/or final composition may be a dry composition.

A dillapiol compound of the present invention is incorporated into a pest control composition at a concentration that will deliver enough dillapiol compound to increase the efficacy of the pest control agent. Those of skill in the art will recognize that the ratio of pest control agent to dillapiol compound will depend a great deal upon the nature and type of the pest control agent which is present in the composition. Typically, however, the weight ratio of pest control agent to dillapiol compound will be in the range from about 0.1:1 to 1:100 and preferably is in the ratio of 1:5

In addition to the aforementioned components, the compositions of the present invention may also contain other pest control agent adjuvants commonly employed in the art. Examples of such adjuvants include crop oil concentrates such as AGRIDEX™, spreaders such as ORTHO™ X-77,

drift control agents, such as LO-DRIFT™, defoaming agents, such as D-FOAMER™, compatibility agents, such as E-Z MIX™, and other adjuvants well known in the pest control agent art. It is understood by those skilled in the art that the amount of these adjuvants in the pest control agent composition can vary widely, and that the amount needed can be readily determined by routine experimentation.

For any given pesticide, the skilled artisan will readily arrive at a pest control composition having the optimum ratio of ingredients by routine experimentation.

5.9 **Advantages**

The use of dillapiol compounds in the method of this invention presents an improvement over previously known methods of treating MDR for a number of reasons. Dillapiol and its analogues are powerful chemosensitizers that inhibit the ABC superfamily of transport proteins; thus, they are promising chemosensitizers for MDR management in the treatment of disease, pest control, and addiction therapies. In addition to this, dillapiol compounds are selectively toxic to MDR cells. This collateral sensitivity is valuable: dillapiol compounds act on the MDR phenotype, not by inhibiting a physiological function found in both healthy and cancerous cells. The fact that dillapiol compounds both exhibit collateral sensitivity to MDR cells and are chemosensitizers makes them ideal candidates for chemosensitizers in chemotherapy.

Since dillapiol compounds are highly toxic to MDR cells, but not to drug-sensitive cells or to non-cancerous cells, these compounds can be used at lower concentrations than other chemosensitizers: their effectiveness is augmented by their specific toxicity to MDR cells. For example, the selective effect of dillapiol on MDR cells was obtained with much lower concentrations than with the standard P-gp inhibitor, verapamil, as illustrated in Figure 2. The use of lower concentrations of chemosensitizers avoids unacceptable side effects, a problem associated with many of the chemosensitizers currently available.

Dillapiol compounds increase the efficacy of administered drugs. This results in the reduction in concentrations of drugs involved in the treatment of diseases and, consequently, a reduction in side effects to drug treatments.

Dillapiol compounds also enhance the activity of pest control agents, decreasing the concentrations of these agents needed to be effective. This is extremely advantageous considering that the use of chemical pest control agents presents the disadvantages of polluting the environment, creating potential hazards to agricultural workers and consumers, and causing detrimental effects on non-target species.

A further advantage of dillapiol compounds is that they exhibit low toxicity to normal, unresistant cells. The cytotoxicity of other chemosensitizers to healthy tissues limits the therapeutic use of many of these compounds. Dillapiol and the closely-related phenylpropanes, however, have been identified in several widely consumed food substance. This indicates that they are well tolerated in the human digestive system. For example, dillapiol is a major constituent of dill and celery seeds; estragole is used as a flavour in foods and liqueurs and is found in *Piper betle*, which is chewed with betel nuts on a daily basis in Asian countries; myristicin is found in carrots, nutmeg, and celery; elemicin is found in carrots; and eugenol is found in nutmeg, oregano, cinnamon, and cassia; and safrole is found in basil and nutmeg (Harborne and Baxter (ed.) *A Handbook of Bioactive Compounds from Plants* (London: Taylor and Francis, 1993) at 472-488).

Dillapiol compounds also act more rapidly on MDR cells than do other chemosensitizers.

In addition to these advantages, dillapiol is cheap to produce since it can be extracted and purified easily from widely-cultivated plants. It is amenable to practical agrobotanical production and purification. Further, dillapiol compounds can improve the efficacy of low doses of chemotherapy, thereby reducing the cost of drug treatments with very expensive therapeutic agents.

6. EXAMPLES

The present invention is described in further detail in the following non-limiting examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

EXAMPLE I

Toxicity Studies

The results presented in this example demonstrate the resistance levels of two series of MDR cell lines (cell lines selected for MDR as well as cells transfected with the P-gp gene) to chemotherapeutic drugs, a known chemosensitizer, verapamil, detergents, and dillapiol compounds.

Cell Lines:

The following cell lines were used: NIH/3T3 (NIH), established from NIH Swiss mouse embryo cultures, and their MDR derivatives transfected with a human gene coding for P-gp, NIH-MDR-G185 (G185) (Bradley *et al.*, (1988) *Biochem. Biophys. Acta* 948:87-128). Both cell lines were provided by the National Cancer Institute, Bethesda, MD. Three Chinese hamster ovarian cell lines were also used: the parental CHO-AUXB1 (AUXB1) and its two MDR lines successively selected with colchicine CH^RC5 (C5) and CH^RB30 (B30) (Ling *et al.*, (1983) *Cancer Treat. Rep.* 67:869-874) CH^RI10 (I10) revertant, drug-sensitive cells were selected from CH^RC5. These cell lines were kindly supplied by Dr. V. Ling of the Ontario Cancer Institute, Toronto, ON, Canada. Both resistant cell lines have exhibited stable levels of cross-resistance to anthracyclines, etoposide (VP-16), and Vinca alkaloids for several years, with highest levels of P-gp in B30 (Ling *et al.*, (1983) *Cancer Treat. Rep.*

67:869).

Cells were grown in plastic flasks (Falcon) in a humidified atmosphere at 37°C, 5% CO_2. The mouse cells, NIH and G185, were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco BRL) containing glutamine. The hamster cells, AUXB1, C5, and B30, were maintained in alpha minimum essential medium (αMEM, Gibco BRL) containing nucleosides and glycine. All culture media were supplemented with 10% fetal calf serum (Wittaker Bioproducts), penicillin (50 units/ml), and streptomycin (50 µg/ml) (Gibco BRL). Resistance levels were maintained with the addition to the cultures of colchicine at the following concentrations: 0.06 µg/ml in G185, 15 µg/ml in C5, and 30 µg/ml in B30.

Determination of Resistance Levels:

A screening of the toxic doses of various test compounds to the cell lines was performed. Adherent cells were detached with a solution of trypsin (0.5% in PBS with 0.2% EDTA), centrifuged at 1000 rpm for 15 minutes, and resuspended in medium to a final concentration of 500 cells/ml. One ml of the cell suspension was distributed in each well of a 24-well plate (Falcon), already containing one ml of the test compound at the appropriate concentration (at least six concentrations). Control wells without test compound contained cells and the solvent (0.1% H_2O:EtOH or DMSO) used to stabilize the test compound. Cells were incubated for eight days at 37°C, 0.5% CO_2. The media were then discarded, and cell colonies were stained with 0.25% methylene blue (in 50% EtOH) for 15 minutes The concentration beyond which cell growth ceased was used for the assessment of the toxicity of the compounds. For a more accurate calculation of the minimum inhibitory concentrations (MIC) of the test compounds, a similar experiment was set up using larger 3.5 mm diameter sterile petri dishes at two different concentrations of cell: 500 cells/3 ml and 5000 cells/3 ml Drug concentrations clustered around the killing dose previously determined in the 24-well plates. Colonies were counted, and cell growth was expressed as percent growth of the respective controls. The results are presented in Table 1.

The resistance levels of the cell lines were calculated as the LD_{50} of the test compound for the cell line of interest divided by the LD_{50} of the test compound for the parental cell line. The lethal concentration of the drugs colchicine, actinomycin D, vincristine, and vinblastine were higher for the resistant cell lines C5 and B30 than for their parental drug-sensitive line AUXB1. Similarly, the lethal concentration of these drugs was 10- to 100-fold higher for the transfected MDR cell line G185 than for its parent NIH.

The chemosensitizer verapamil was equally toxic to both the parental and MDR cell lines.

The MDR cell lines showed collateral sensitivity to the detergents NP-40, Triton X100, and phenolic DBP, but not to Tween 80. This phenomenon was eliminated in revertant, drug-sensitive I10 cells, indicating that both hypersensitivity and resistance are an intrinsic part of the MDR phenotype in these cell series.

Table 1. Effect of Drugs and Other Agents on the Resistance Level[1] of Cancer Cell Lines

	CELL LINES					
	Murine human MDR1 (transfected)		Hamster treated with colchicine (selected)			
	NIH sensitive	G185 MDR	AUXB1 sensitive	C5 MDR	B30 MDR	110 revertant
	1	25	1	96	680	28
	1	60	1		>4	
	1	100	1		75	
	1	10	1		50	
MDR Group of Drugs:[2]	1	1	1		1	
colchicine						
actinomycin D			1	1.3	0.9	1.2
vincristine			1	0.6	0.4	1.1
vinblastine			1	0.2	0.06	1.1
Chemosensitizer for MDR: Lignans and Derivatives:[4]			1	0.5	0.09	1.2
podophyllotoxin	1	9.2	1		100	
VP-16	1	10	1		10	
dillapiol	1	0.61	1		0.001	
piperonyl butoxide	1	0.1	1		0.01	

[1] Resistance Level = LD_{50} resistant cell line / LD_{50} sensitive parental cell line

[2] P. Juranka, personal communication.

[3] Detergents showing higher toxicity to MDR than sensitive cells: Loc and Sharom (1993) *Br. J. Cancer* 68:342-351.

[4] Bourret-Bernard (1995) PhD thesis, University of Ottawa.

Both MDR cell types, selected and transfected, showed collateral sensitivity to dillapiol and one of its synthetic analogues, piperonyl butoxide (see Figure 4). Both compounds were at least 10 to 100 times more toxic to MDR than to parental cells. The very low concentrations of piperonyl butoxide that were effective (0.5 and 5 ng/ml with B30 and G185, respectively) are far below the concentrations found in currently commercialized insecticidal mixtures. Similarly, the very low MIC of dillapiol (0.5 ng/ml with B30) is far below the concentration found in plants, in particular the *Piperaceae*.

EXAMPLE II

Drug Accumulation Studies Using Vinblastine

The effects of dillapiol compounds on the net accumulation in cells of the drug vinblastine is shown. Cell lines used were the same as described in Example I.

This example was carried out at room temperature following a method modified from Lemontt *et al.*, (1988) *Cancer Res.* 48:6348). In short, trypsinized cells were washed and resuspended in PBS-glucose (10 mM), at a concentration of 1.5 X 10^6 cells/ml. A 1 ml cell suspension was mixed with 2 µl ^{3}H-vinblastine/ml (specific activity 11 Ci/mM), with or without the potential resistance modifying compounds. At specific intervals (1, 15, 30, 60, and 90 minutes), 300 µl of the mixture was layered onto 250 µl of a silicone oil:mineral oil mixture (4:1, v:v, Aldrich silicone oil, d=1.05:Fisher light mineral oil), and immediately centrifuged 10 seconds at 500x*g* on a countertop centrifuge. The aqueous phase was removed, the upper part of the tube rinsed with 500 µl PBS to eliminate risks of contamination of the pellet with the unincorporated radioactivity, the oil removed, and the pellet digested overnight in 500 µl NaOH (0.1N). Five ml of scintillation liquid was added to the pellet; counting of tritium activity was done on a Beckman LS1701 scintillation counter.

The validity of the system was established by using verapamil (15 µM), a specific P-gp inhibitor, as a positive control in each experiment.

Accumulation of ^{3}H-vinblastine correlated with the resistance level of the 5 cell lines studied (Figure 1). A plateau was reached after 60 minutes in all cell types. There was 2.5-fold more vinblastine in NIH cells than in the MDR cells G185; the hamster drug-sensitive AUXB1 cells had 2.5-fold more vinblastine than C5 cells and 4.2-fold more than B30 cells. These counts represent the net cellular loading that results from the combination of the uptake of the drug by cells, minus its active efflux by the membrane bound P-gp pump. Counts were stable from one experiment to the next once the proper protocol was established. The results were comparable to results reported from other studies using vinblastine and other drugs recognized and transported by the P-gp pump.

Verapamil, a specific inhibitor of P-gp, reverses the MDR phenotype (Lemmont *et al.*, (1988) *Cancer Res.* 48:6348-6353; Loe and Sharom (1993) *Br. J. Cancer* 68:342-351). It restored the level of vinblastine accumulated by B30 cells to that of the drug-sensitive AUXB1 cells (Figure 2) Murine cell responses to verapamil paralleled that of hamster cells (data not shown).

Dillapiol had a larger effect on the net accumulation of ^{3}H-vinblastine in the MDR hamster cells (B30) than on the drug-sensitive cells (Figure 2). While the lower doses (9 nM and 18 nM) had little modulating effect on the net uptake in sensitive cells, they increased the net uptake in resistance cells by five times (DPM in B30 without dillapiol = 1800 ± 4000; with dillapiol = 102,000 ± 9000). Such results are consistent with toxicity studies showing a hypersensitivity of MDR cells to dillapiol (Table 1).

The MDR-selective modulating effects of dillapiol on the ^{3}H-vinblastine uptake were more rapid and obtained with much lower concentrations than with verapamil. After a 15 minute incubation of B30 cells, the net accumulation of vinblastine in dillapiol-treated cells was three times that of vinblastine in verapamil-treated cells (Figure 2). None of the doses assayed were toxic to the drug-sensitive cell line AUXB1 (MIC for dillapiol = 500 ng/ml = 2.2 μM).

A variety of dillapiol derivatives were shown to have a similar effect on net accumulation of ^{3}H-vinblastine in the MDR hamster cells, B30 (Figure 3).

EXAMPLE III

Drug Accumulation Studies Using α-Terthienyl

The effects of dillapiol compounds on the net accumulation in cells of the drug α-terthienyl was investigated.

Chemicals:

Dillapiol was purifed and kindly provided by Dr. Krishnamurty (India) Alpha-terthienyl was used from a stock that was synthesized as described in Philogene *et al.*, (1985) **[Complete cite needed]**

Effects of Dillapiol on the Accumulation of α-terthienyl in Mosquito Larvae:

Mosquito larvae *Acdes atropalpus* (Diptera: Culcidae) were incubated in the presence of a constant sub-lethal concentration of α-terthienyl, 1 µg/ml, and in the presence of variable concentrations of dillapiol ranging from 0 to 1 µg/ml. The amount of α-terthienyl in the tissues was then determined.

Fourth instar larvae (13-21 larvae per replicate, 4 replicates per treatment) were incubated in 7-ml glass vials containing 5 ml of distilled water to which α-terthienyl had been added at a concentration of 1 µg/ml, through 20 µl of ethanol solution. All treatments received the same amount of ethanol. Larvae were then incubated for 24 hours in darkness to prevent the photosensitization processes mediated by α-terthienyl when exposed to near-UV wavelengths. Under these specific conditions, no larval mortality was observed in any treatment.

At the end of the incubation period, the larvae were slightly dried out on a paper towel, then

transferred in vials conatining 2 ml of hexane and incubated with agitation in darkness for 3 days. Preliminary experiments showed that the yield of this extraction protocol of α-terthienyl was higher than 92%. An aliquot of 1 ml of hexane was then sampled for HPLC analysis and the larval tissues were put in an oven at 55°C for 24 hours to determine the dry weight.

HPLC Analysis of α-Terthienyl

Alpha-terthienyl was analyzed by HPLC using acetonitrile:water (7:3) at a flow rate of 1 ml/min with a C-18 reverse phase column (250 mm * 4.6 mm, Beckman, USA). An HPLC Beckman Gold System including a solvent module (model 126), a UV-detector (model 168), and an autosampler (model 502) was used. Under these conditions, the retention time of α-terthienyl was 11.4 min. The specific peak areas on the chromatograms were used to determine the concentration of α-terthienyl. A calibration curve was made with pure α-terthienyl diluted to different concentrations in hexane: 0.2, 1.0, 5.0, 10.0, and 25 µg/ml. The coefficient of determination values (r^2) according to the peak areas measured was 0.994. The concentration of α-terthienyl in mosquito larvae was expressed in µg of α-terthienyl per gram of dry weight larval tissues.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various usages and conditions. Such changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims

The embodiments of the invention in respect of which an exclusive property or privilege are claimed are as follows:

1. A method for inhibiting or controlling MDR, said method comprising administering to a patient in need of inhibition or control of MDR an effective amount of one or more compounds selected from a class of dillapiol compounds comprising dillapiol, its derivatives and its analogues, wherein said compounds are selected on the basis of their ability to exert a negative effect on MDR.

2. The method according to claim 1, wherein said compound is administered as a pharmaceutical composition, said pharmaceutical composition comprising one or more compounds of claim x and an acceptable pharmaceutical carrier.

3. The method according to claim 1, wherein said compound is dillapiol.

4. The method according to claim 1, wherein said compound is piperonyl butoxide.

5. A method of adversely affecting MDR cells in pests, comprising administering to the pest an effective amount of one or more dillapiol compounds, wherein the dillapiol compound is selected from the group consisting of dillapiol and analogs, salts, or derivatives thereof, and combinations thereof, wherein said compounds are selected on the basis of their ability to exert a negative affect on said MDR cells in pests.

6. A method of sensitizing MDR cells in a subject, comprising coadministering to the subject an effective amount of one or more dillapiol compounds and an effective amount of a therapeutic agent, wherein the dillapiol compound is selected from the group consisting of dillapiol and analogs, salts, or derivatives thereof, and combinations thereof, wherein said compounds are selected on the basis of their ability to exert a negative affect on said MDR cells in mammals.

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Vinblastine uptake

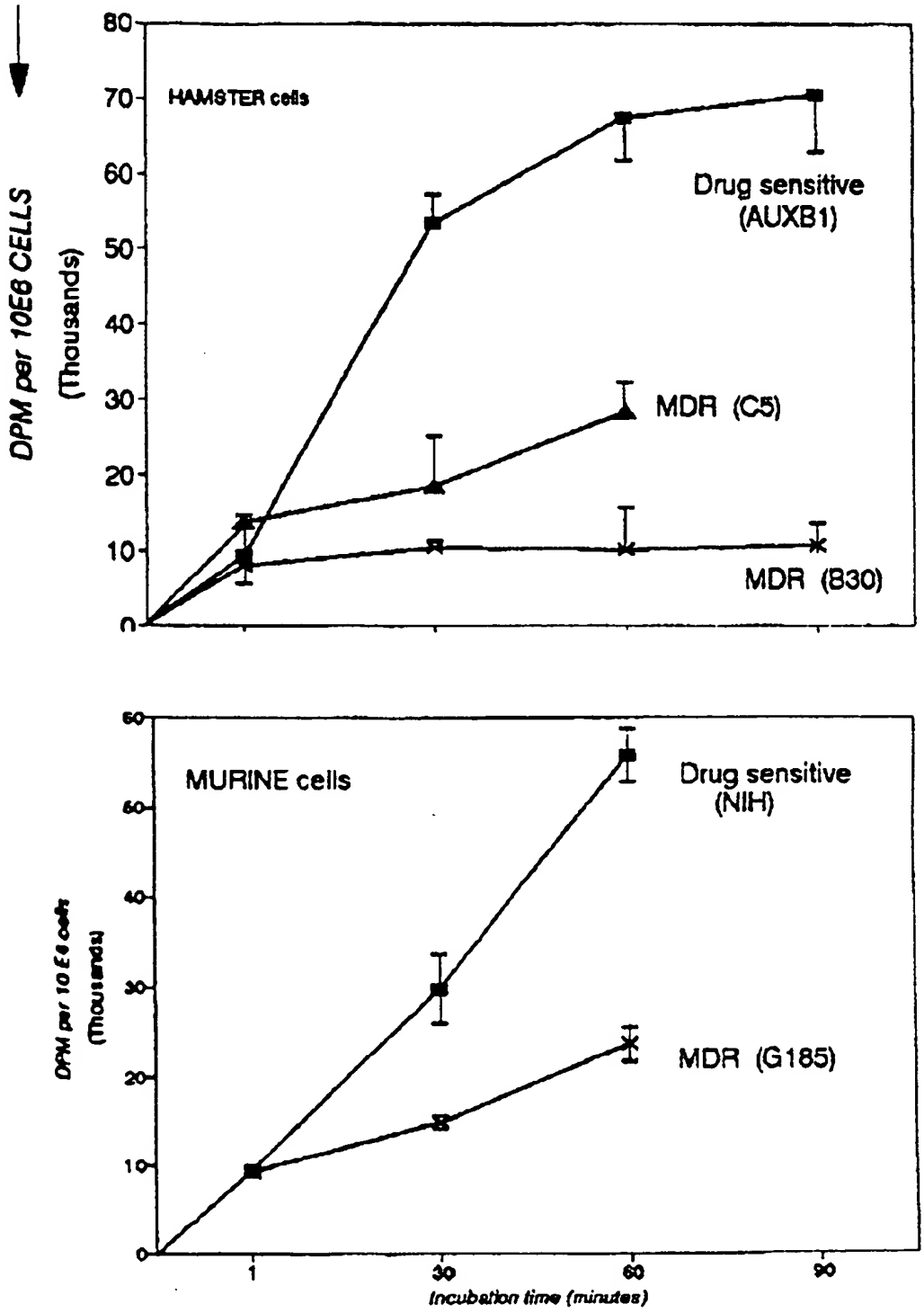

Figure 1 of 5

– Vinblastine uptake

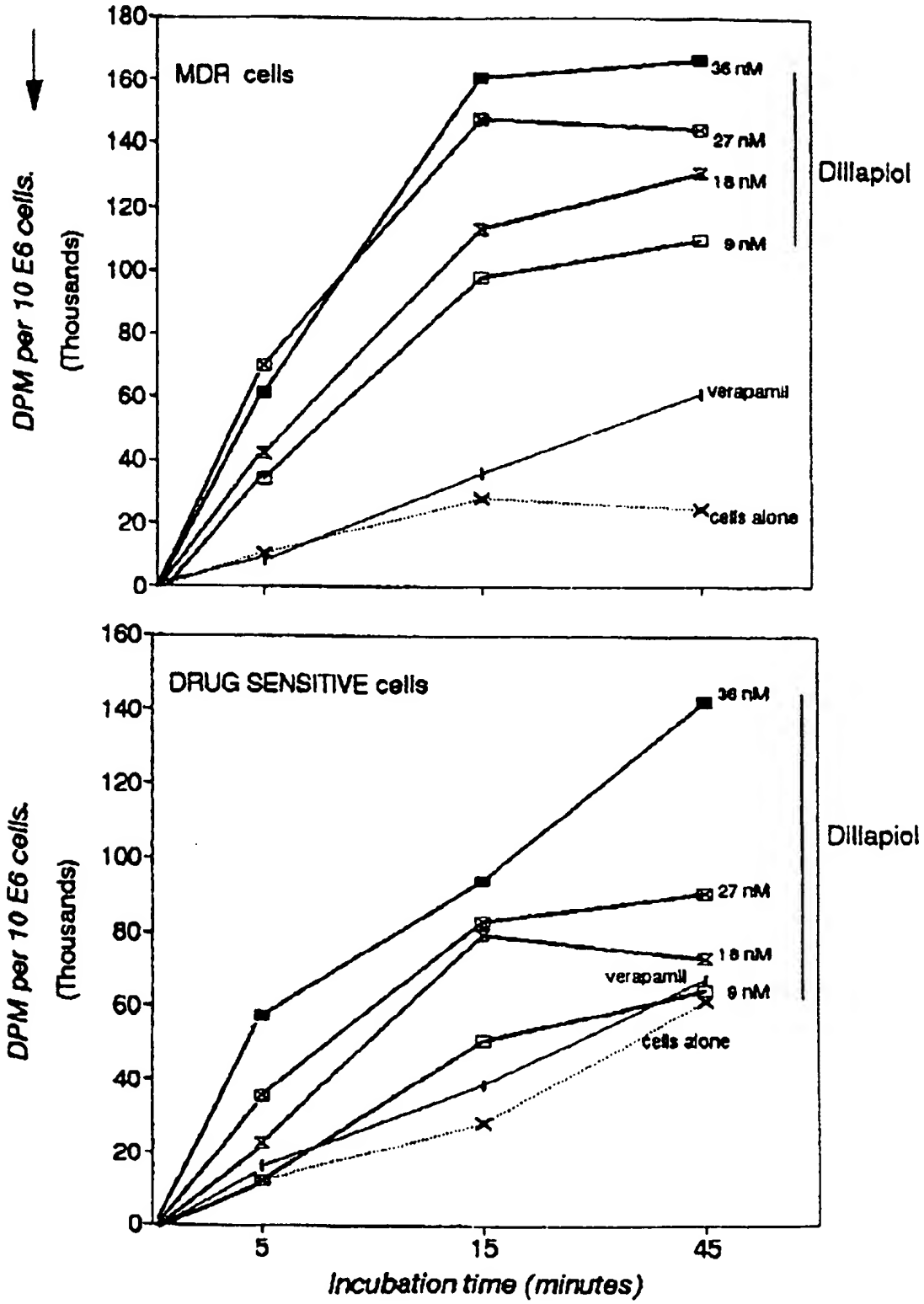

Figure 2 of 5

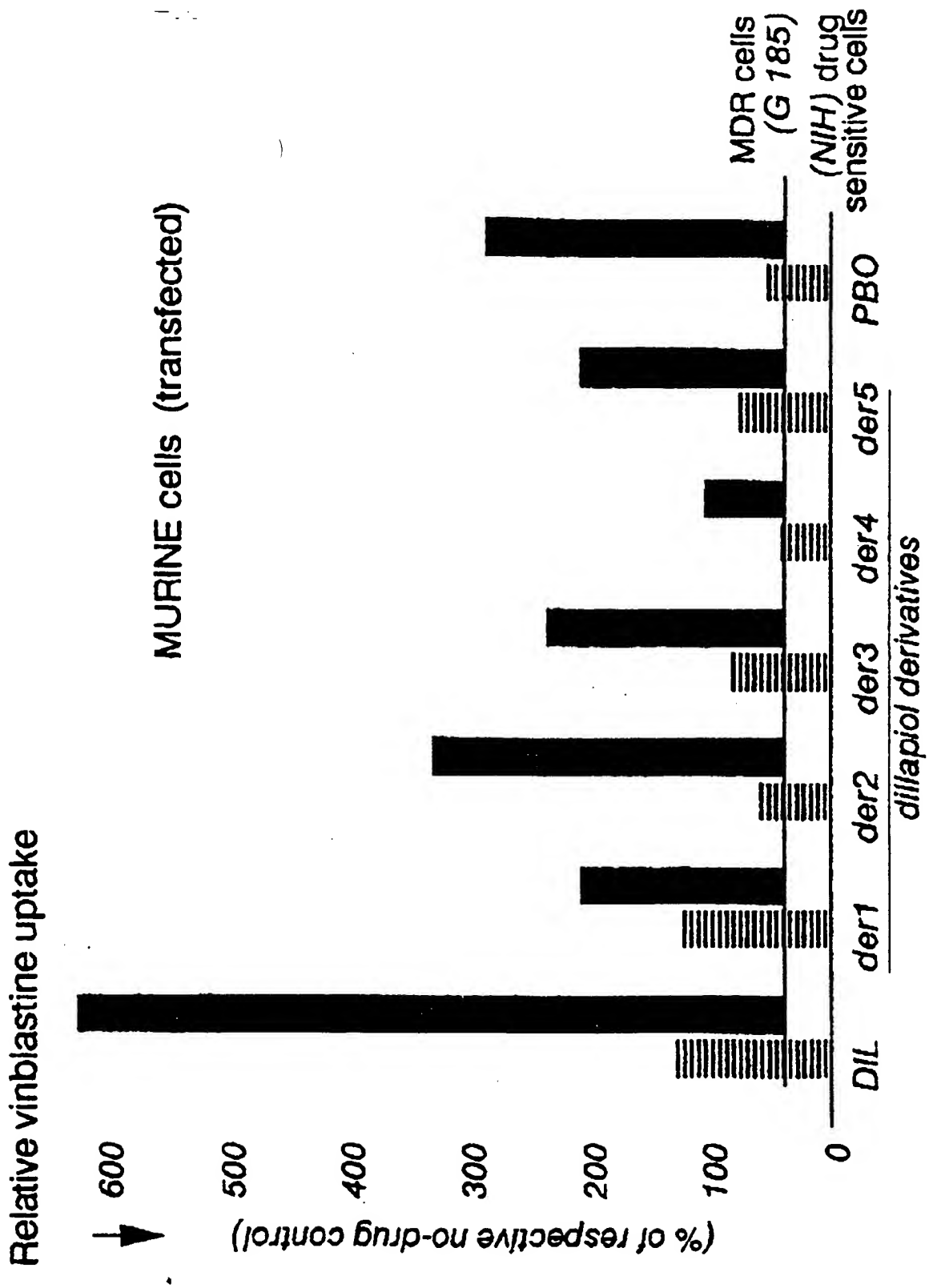
Relative vinblastine uptake
(% of respective no-drug control)
600
500
400
300
200
100
0
MURINE cells (transfected)
MDR cells (G 185)
(NIH) drug sensitive cells
DIL
der1
der2
der3
der4
der5
PBO
dillapiol derivatives

Figure 3 of 5

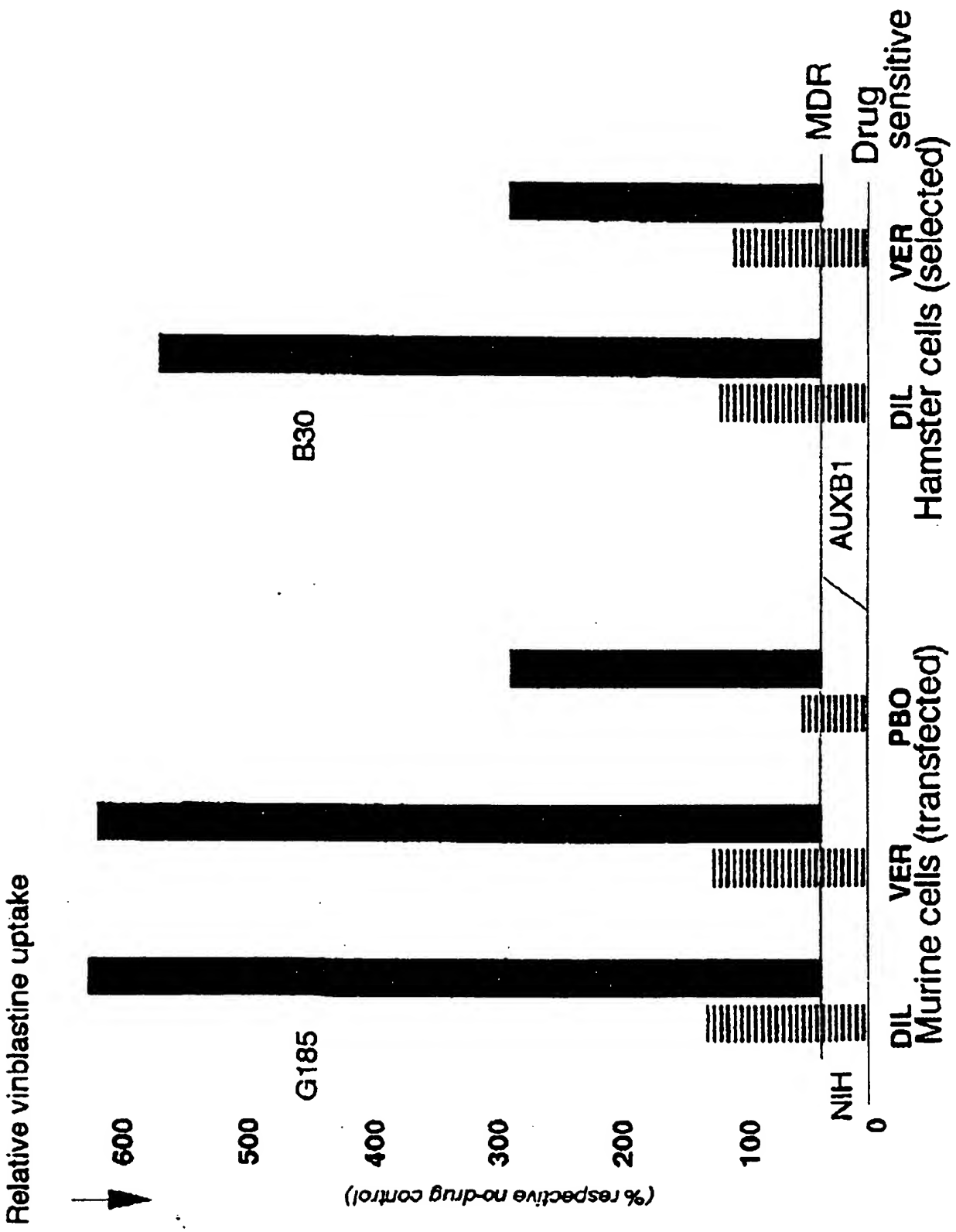
Relative vinblastine uptake
(% respective no-drug control)
600
500
400
300
200
100
0
G185
B30
AUXB1
NIH
DIL
VER
PBO
DIL
VER
MDR
Drug sensitive
Murine cells (transfected)
Hamster cells (selected)

Figure 4 of 5

Effects of dillapiol on the accumulation of α-terthienyl in the tissues of 4th instar mosquito larvae, *Aedes atropalpus* (Diptera: Culicidae).

Mosquito larvae were incubated in water with α-terthienyl (50 ppb) and dillapiol for 24 hours in darkness.

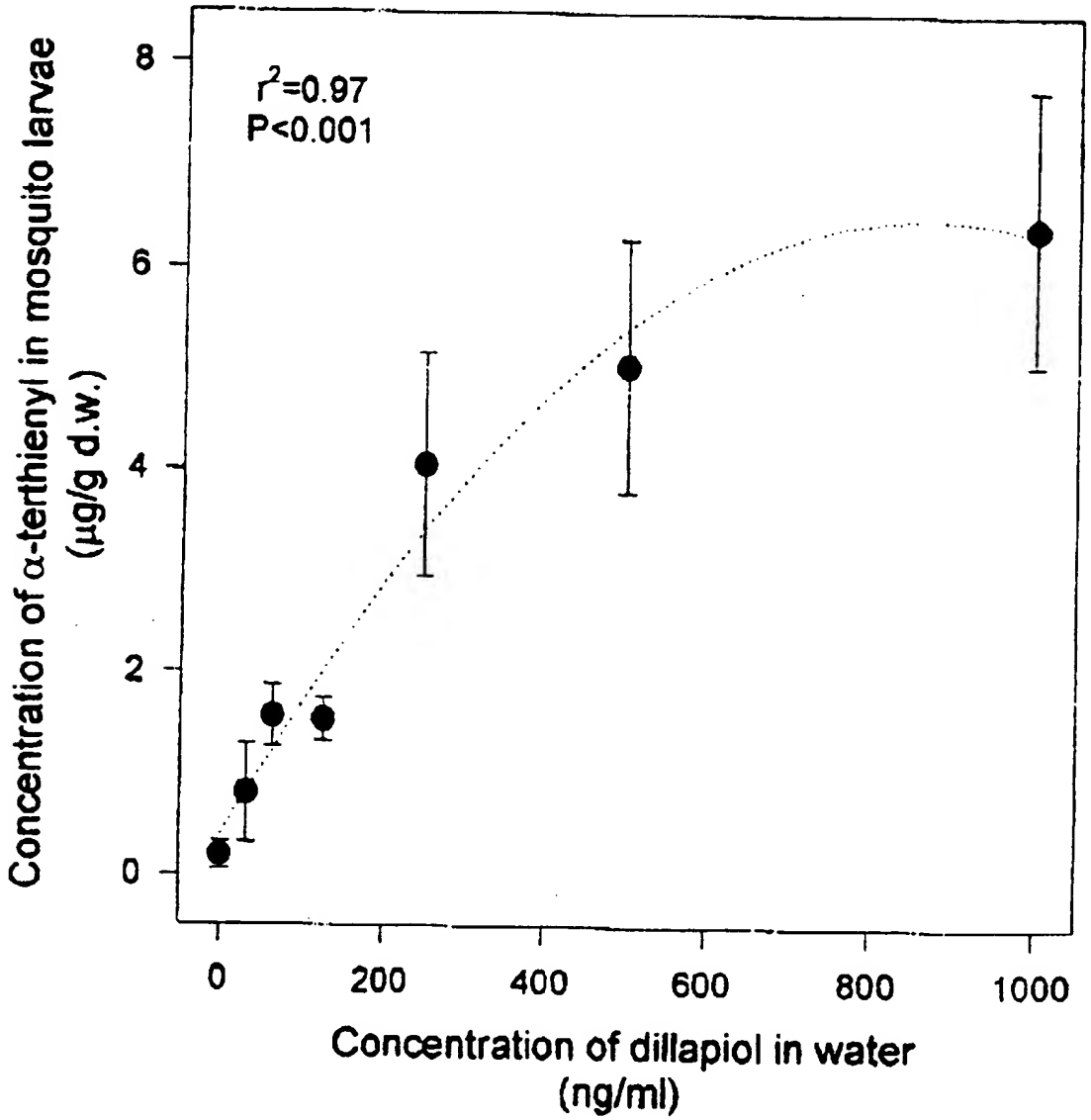

Figure 5 of 5